VIROLOGICAL SURVEY OF SELECTED BATHING SITES AND SEWAGE TREATMENT PLANTS IN SOUTHERN ONTARIO

PHASE II REPORT

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Ministry of the Environment

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VIROLOGICAL SURVEY OF SELECTED BATHING SITES AND SEWAGE TREATMENT PLANTS IN SOUTHERN ONTARIO - PHASE II REPORT

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SYNOPSIS

An adsorption-elution method using Duofine membrane filters for the concentration of viruses from water, was refined for use in the Ontario Ministry of Environment Laboratory and was subsequently used to process 40 L samples of surface water and sewage effluent from selected sites in Southern Ontario. A qualitative gauze-pad method was refined and used for the analysis of influent and effluent sewage samples.

Virus positive samples were detected by the occurrence of viral cytopathic effects (CPE) in cell cultures and the presence of virus was confirmed by electron microscopy. Some of the viral isolates have been tentatively identified, based on the type of CPE observed, and on the size and shape of the virus particles as viewed under the electron microscope.

Twenty surface water samples were collected from selected bathing beach sites on the Great Lakes. No viruses were isolated from any of these samples. Eleven sediment samples from these sites were also analyzed and no viruses were detected.

Three of seven surface water samples collected from two bathing sites on rivers in Southern Ontario tested positive for viruses. The source of these viruses is unknown.

A total of 69 samples were collected and analyzed from four sewage treatment plants and their receiving waters. Viruses were isolated from 37 (54%) of these samples.

Electron microscopic examination of some of the viruses isolated has revealed the presence of enteroviruses and reoviruses.

Evidence is presented which confirms that sewage treatment plant effluents are point sources of enteric viruses in the environment, and that conventional sewage treatment practices, including chlorination, are insufficient

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for the 100% removal of viruses from sewage. Thirteen of sixteen (81%) non-chlorinated effluent samples were virus positive, and six of thirteen (46%) chlorinated effluent samples were positive for virus.

Further studies are necessary in order to determine the origin and significance of viruses isolated in this study.

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OBJECTIVES

1. To evaluate and refine available methods for detecting and isolating viruses from surface waters, sediments, and influent and effluent sewage.
2. To perform virological analyses on specified sewage influents, treated effluents, and on the receiving waters for these effluents.
3. To perform virological analyses on water and sediment samples from selected bathing beaches on the Great Lakes.
4. To perform virological analyses on water samples collected from selected bathing beaches on inland rivers.
5. To prepare a report summarizing the results of the analyses with conclusions and recommendations.

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INTRODUCTION

Over 100 types of enteric viruses are known to be excreted in human feces (1). As many as 10^9 virus particles may be excreted per gram of feces and 10^5 infectious virus particles per litre of raw sewage have been detected (1, 2). The amount of virus present in raw sewage at any given time is highly variable, depending primarily on the extent of viral disease in the community and the percentage of domestic waste in the raw sewage. Peak enterovirus levels usually occur in late summer and early fall, while lower densities are normally prevalent during the rest of the year (1, 3, 4, 5).

Several studies conducted in developed countries have demonstrated enteric viruses in sewage effluents, contaminated streams, rivers, lakes, and drinking water (1, 6, 7, 8, 9). Enteric viruses may easily survive secondary sewage treatment and chlorination, as commonly practised, and thereby may be discharged into natural waters where they can persist for many months (1, 6, 10, 11). Since enteric viruses are considerably more resistant to various sewage and water treatment methods than either coliform or other enteric bacteria, the absence of such bacterial sanitary indicator organisms does not guarantee the absence of a viral disease hazard (11). Water contaminated with enteric viruses may pose a health hazard when it is used for drinking, recreation, or irrigation.

Enteric viruses have been identified as the etiological agents responsible for waterborne outbreaks of disease. Waterborne outbreaks caused by hepatitis A virus are well documented (1, 12), and adenovirus infections have been recognized among persons frequenting contaminated swimming pools (13). Recently, coxsackievirus B5 was isolated from an open lake swimming area during an outbreak of mengingitis at a summer camp for boys (1, 9). Coxsackievirus B5 was identified as the causative agent of the outbreak. Conclusive evidence for the waterborne transmission of other enteric viruses is not available. This is primarily

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due to limitations of the methods used for recovering enteric viruses, the difficulties involved in assessing inapparent infections in the population, and a lack of extensive epidemiological studies. Although one plaque forming unit (PFU) of virus is considered capable, under ideal circumstances, of initiating infection in man, most enteric virus infections result in inapparent or subclinical infections which are usually unreported and make epidemiological studies difficult to conduct (1, 2, 10, 11, 14, 15). Even if infections are apparent, the broad spectrum of disease syndromes caused by enteric viruses makes it difficult to attribute scattered cases of illness to a specific etiological agent unless intensive and costly diagnostic procedures are employed (1). In addition, suitable in vitro methods for the isolation of some of the enteric viruses of most concern (e.g. hepatitis A virus, rotavirus and Norwalk agent), are presently not available. In fact, only one-third of the enteric viruses known can be cultivated in vitro using current methods (1). Considering all of these factors, the presence of even one detectable infectious virus unit in water may pose a potential health hazard, and the absence of detectable virus does not necessarily preclude the existence of undetectable viruses.

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MATERIALS AND METHODS

A. GROWTH MEDIA

Eagle's minimal essential medium containing Earle's salts (MEM E) was supplemented with gentamicin (50 ug/ml), sodium bicarbonate (11 ml of 5.6% $NaHCO_3$ per 500 ml of medium), and L-glutamine (2 mM/ml). Fetal calf serum (FCS) was added to the supplemented MEM E to give a final concentration of 5% FCS for passaging cells and 2% FCS for maintenance media.

B. CELL CULTURES

BS-C-1, a continuous cell line of African green monkey kidney cells, was used for the isolation and propagation of virus throughout the study. Human embryonic kidney (HEK) cells, a primary cell line, were used in conjunction with the BS-C-1 cells for all samples analyzed after March, 1980. Stock cultures of cells were grown as monolayers in 75 cm^2 plastic tissue culture flasks using MEM E containing 5% FCS, gentamicin and L-glutamine. The cell monolayers were normally confluent after 3-4 days of incubation at 37°C. To passage cells, the monolayers were trypsinized with trypsin (0.25%)-versene (0.05%) in phosphate buffered saline at 37°C for 5-15 minutes. The cells were resuspended in growth medium at a split ratio of 1:3 and inoculated into 75 cm^2 and 25 cm^2 flasks.

C. VIRUS

Type 1 poliovirus, Sabin strain, was employed for the evaluation of the recovery efficiency of the virus concentration method. The virus was obtained from the Ministry of Health, Laboratory Services Branch. It was passaged in BS-C-1 cells, dispensed into glass vials in 5 ml aliquots, and stored at -70°C.

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D. CONCENTRATION OF VIRUSES FROM WATER AND WASTEWATER BY MEMBRANE FILTRATION

The method employed in this study was a modified version of the proposed standard method for the detection of enteric viruses in water and wastewater (16). The procedure involves five major steps: a) Sample conditioning; b) Primary concentration; c) Primary elution; d) Secondary concentration; and e) Secondary elution.

1. Procedure for concentrating viruses from samples of surface water and wastewater

i) Sample Collection and Conditioning

Forty litre grab samples of surface water or sewage effluent were collected in two 20 L collapsible plastic containers which were used only once to avoid cross-contamination of samples. In order to enhance the adsorption of viruses to the filters, each sample was then "conditioned" by: 1) adding Earle's balanced salt solution (EBSS), a source of cations, to a final concentration of 1:100; and 2) adjusting the pH of the sample to 5.5 - 6.0 with 1N hydrochloric acid (HCl). In addition, 0.5% sodium thiosulphate was added to a final concentration of 1:100 to all of the sewage effluent samples in order to neutralize any free chlorine. Sodium thiosulphate was added to both chlorinated and unchlorinated effluents in order to preserve uniformity of sample treatment.

ii) Primary concentration

Virus particles were concentrated by filtration through pleated cartridge membrane filters. The "conditioned" sample was filtered under gravity through two melamine-fiberglass pleated membrane cartridge filters (Duofine series, Filterite Corp. Timonium, Md.): a 3.0 µm porosity filter which served as a prefilter; and a second filter of 0.45 µm porosity which was connected to the first.

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When all of the sample had been processed, the filters' housings were disconnected from the reservoir and the fluid remaining in the housings was poured out. The outlets of the housings were sealed with parafilm and the filters, in their housings, were placed on ice, in coolers, for transportation to the laboratory where they were stored overnight at $4^{o}C$.

The collection, conditioning, and primary concentration of samples was generally carried out in the field. However, in poor weather conditions, some water samples were chilled on ice and brought back to the laboratory for complete processing.

iii) Primary elution

Residual water in the filter housings was removed with air pressure and was discarded. Adsorbed virus was eluted from the filters with 800 ml of freshly prepared 0.05 M glycine buffer, pH 11.5, containing phenol red (0.0005%) as a pH indicator. The glycine buffer was passed through the prefilter and then through the 0.45 μ filter. It was permitted to remain in contact with each of the filters for about 1 or 2 minutes before being forced out of the filter housings through the use of positive air pressure. The glycine buffer eluate from the prefilter was re-adjusted to pH 11.5 with 1N NaOH before being used to elute from the second filter. After both filters had been processed, the eluate was neutralized immediately with 0.05 M glycine buffer (adjusted to pH 1.0 with 12 N HCl) in order to minimize inactivation of virus due to prolonged exposure to high pH.

iv) Secondary concentration

In order to enhance virus adsorption during reconcentration, the pH of the primary eluate was lowered to 3.5 with 0.05 M glycine, pH 1.0. Then 0.05 M aluminum chloride ($AlCl_3$) was added to a final concentration of 0.0005 M. The eluate was filtered under vacuum through a filter bed

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consisting of, from top to bottom, a teaspoon of Celite 503 filter aid (J. T. Baker), a 47 mm diameter AP25 prefilter disc (Millipore Corp.) and a 47 mm, cellulose nitrate membrane (0.45 µm porosity, Millipore Corp.). This filter bed was then washed with 30 ml of 0.15 M sodium chloride (NaCl), pH 3.5, to remove residual $AlCl_3$.

v) Secondary elution

The virus was eluted from the filter bed with 8 ml of 0.05 M glycine, pH 11.5 containing 2% FCS. The eluate was passed through a 25 mm, 0.45 µm porosity membrane (Millipore Corp.) to remove bacterial and fungal contaminants, and neutralized immediately with sterile 0.5 M glycine, pH 1.0. The final volume of the concentrate was approximately 10 ml and the entire volume was subsequently assayed for virus.

E. ASSAY OF SAMPLE CONCENTRATES FOR VIRUS

The sample concentrates were assayed for virus in the BS-C-1 and HEK cell cultures described earlier. The growth medium from confluent monolayers of cells grown in 25 cm^2 flasks was removed and discarded. The flasks were then inoculated with 1 ml of the concentrated sample. Ten replicate flasks were used for each concentrated sample. The inoculated flasks were incubated at 37°C for 60 minutes to allow virus adsorption to the cells. After adsorption, 5 ml of maintenance medium (MEM E containing 2% FCS) was added to each flask and the cell cultures were incubated at 37°C. Uninoculated cell culture flasks were included as controls.

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1. First passage

The cell cultures were observed microscopically for viral cytopathic effects (CPE): daily for the first three days; and then periodically for a total of 14 days. Cell cultures exhibiting CPE were frozen when 75-100% of the cell monolayer was affected. Cell cultures negative for CPE after 14 days of incubation were also frozen.

2. Second passage

Cell cultures positive for CPE on first passage routinely underwent a second passage. Cell cultures from the first passage were thawed and the harvest fluids pooled. CPE positive and CPE negative cell cultures were pooled separately. Twenty percent of each pooled harvest-fluid was inoculated, in 1 ml aliquots, onto BS-C-1 and HEK cells and monitored for CPE as described previously. Cell cultures negative for CPE after 14 days were considered to be negative for virus. Cell cultures positive for CPE and those which exhibited a questionable CPE underwent a third passage. Cultures negative for CPE after the third passage were considered to be negative for virus. All samples exhibiting CPE on third passage were suspected positive for virus, and cell lysates were prepared for electron microscopic examination.

F. PREPARATION OF SAMPLE FOR ELECTRON MICROSCOPIC EXAMINATION

1. Preparation of sample aliquot

Tissue culture flasks containing suspected positive samples (see E. 2.) were frozen after 75-100% of the monolayer had been affected by CPE. The flasks were thawed and a small volume of fluid (approx. 2 ml) was removed and centrifuged for 10 minutes at 5000 rpm to remove cellular debris. The supernantants were recovered and prepared for electron microscopic examination.

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2. Preparation of grid

Three hundred mesh, carbon-coated, Formvar grids were placed on 1% Noble agar plates. One to two drops of the supernatant were placed on each grid and allowed to air-dry under ultraviolet light for 20-30 minutes. The grids were removed, placed on a sheet of parafilm, and one drop of 2% phosphotungstic acid stain (2% PTA in 1N KOH, pH 6.5, containing a few grains of bovine serum albumin) was deposited on each grid. After one minute, the PTA stain was removed using filter paper and the grids were examined in a Siemens Elmiscop 102 electron microscope for the presence of virus.

G. IDENTIFICATION OF VIRAL ISOLATES

In some cases, a preliminary indication of the viral group was obtained from the characteristic CPE observed in infected cells, and from the length of time required for 100% CPE to occur. Electron microscopy was then used to tentatively identify the viral group of the isolates where possible. Only the first set of Ingersoll samples have been positively identified at present.

H. PROCEDURE FOR DETERMINING THE RECOVERY EFFICIENCY OF THE CONCENTRATION METHOD

An aliquot of stock poliovirus type 1, Sabin, which had been stored at -70°C, was thawed at room temperature. The virus was diluted to the desired concentration in MEM E and then added to a "conditioned" (See D.1.) sample of either deionized or lake water. The virus was concentrated by the filtration method as described previously. Dilutions of the final, concentrated eluate were prepared in MEM E (2% FCS) and 1.0 ml aliquots were inoculated into flasks containing BS-C-1 cells. The cell cultures were observed for 7 to 10 days and the titres of virus recovered were calculated by the $TCID_{50}$ method. A virus control,

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consisting of dilutions of stock virus in MEM E was used to determine the actual titre of the input virus. The percentage of input virus recovered was then determined for each experimental sample and was used to express the efficiency of the concentration method.

I. REGENERATION OF DUOFINE PLEATED MEMBRANE FILTERS

The Duofine filters could be regenerated and reused up to three times before the flow rates were reduced significantly. For regeneration, the filter housings containing the "contaminated" filters were filled with 800 ml of 1N NaOH and left to stand at room temperature for a minimum of two hours. This alkaline solution was back-flushed out of the housings with positive air pressure. This washing process was repeated with 0.04 N HCl and with deionized water. Finally the filters were sterilized by autoclaving at 110°C for 15 minutes.

J. CONCENTRATION OF VIRUSES FROM SEWAGE BY THE GAUZE-PAD METHOD

1. Preparation of pads and sample collection

Viruses present in sewage may be concentrated by the gauze-pad method, which involves the entrapment of aggregated or particulate-associated viruses. The gauze-pads, measuring approximately 15 cm x 15 cm, consisted of absorbent cotton bound with cheese cloth and tied with a 30 lb fishing line. A No. 12 lead sinker was inserted into the middle of each pad for added weight. The assembled pads were sterilized by autoclaving at 121°C for 15 minutes. During the sampling process, the pads were suspended on a 30 lb fishing line in the sewage flow for 24-48 hours. The influent sewage pads were suspended in the influent pipe before the coarse filter. Effluent pads were usually suspended in the flow at the outlet from the chlorine contact chamber to the effluent pipe. At the time of collection, excess fluid was squeezed out of the pads and

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extraneous accumulated surface material removed. The pads were placed in plastic bags, sealed, and transported on ice-packs, in coolers, to the laboratory where they were stored overnight at 4°C before processing.

2. Processing sewage pad samples

The residual fluid was squeezed out of the pad and poured into a sterile bottle. An eluant consisting of 100 ml of 0.05 M glycine buffer, pH 11.5, containing 10% FCS was added to the plastic bag containing the pad. The pad was soaked and kneaded in the eluting fluid for two minutes. The pH of the fluid was then measured and adjusted, if necessary, to a value of 9.0 - 9.5 with 1N NaOH. The pad was again soaked and kneaded in the eluting fluid for five minutes, with periodic checking to ensure the pH of the fluid was 9.0 - 9.5

Approximately 100 ml of eluate was recovered and poured into a sterile bottle. This eluate was then supplemented with the original residual fluid to a final volume of 200 ml, and the pH of this combined fluid was adjusted, if necessary, to 9.0 - 9.5. The mixture was centrifuged at 14,000 rpm for 30 minutes at 5°C in a refrigerated ultracentrifuge. Ten ml of the supernatant was removed and clarified by filtration through a filter bed consisting of Celite 503, an AP25 prefilter, and a 0.45 µm membrane filter. The clarified supernatant was sterilized through a 0.45 µm membrane and neutralized with sterile 0.05 M glycine, pH 1.0, before being assayed for virus. The remainder of the original supernatant was stored at -70°C.

K. EXTRACTION OF VIRUSES FROM SEDIMENTS

1. Sample collection

Sediment samples were collected in clean, 1 L, wide-mouthed, plastic bottles. The bottle was immersed in the water and run along the surface of the lake bed to collect the top 3 cm of sediment. This process was continued until

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the container was one-half full. The sediment was allowed to settle for a few minutes before the excess water was poured off. The sediments were transported to the laboratory on ice and placed into storage at -20°C until processing.

2. Processing sediment samples

The frozen sediments were thawed and any residual water poured off. Fifty ml of eluting fluid (0.05 M glycine buffer, pH 11.5, containing 10% FCS) was added to the sample and mixed thoroughly by shaking. The sediment was allowed to settle and then the pH of the supernatant was adjusted, if necessary, to a value of 9.0 to 9.5 with 1N NaOH. The sample was again mixed by shaking. The liquid eluate was then recovered and clarified by filtration through a filter bed consisting of Celite 503, an AP25 prefilter, and a 0.45 µm membrane filter. The pH of the filter-sterilized eluate was adjusted to 7.2 - 7.5 with sterile 0.05 M glycine buffer, pH 1.0. Ten ml of this eluate was assayed for virus and the remainder was stored at -70°C.

L. SAMPLING LOCATIONS

1. Beaches

Surface water samples for viral analysis were collected at Jones Beach, Confederation Park, Hanlan's Point Beach, Breakwater Beach, Lakeside Park and Municipal Beach, all situated on Lake Ontario. In addition, samples were collected from Iroquois Beach Provincial Park on Lake Erie, Ipperwash and Grand Bend Beaches on Lake Huron, and Wasaga Beaches #2 and #5 on Georgian Bay, Lake Huron. Maps of the sampling sites at each of these beaches are given in the Phase I report entitled, "A study of disease incidence and recreational water quality in the Great Lakes".

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2. Conservation areas

Surface water samples from bathing beaches on the Humber River were collected from two conservation areas, Boyd and Clairville, located just northwest of Toronto.

3. Sewage treatment plants

Four sewage treatment plants (STP) were sampled during the course of the study: the Ingersoll STP in Southwestern Ontario; the Humber STP in West Toronto; the Port Weller STP in St. Catharines; and the Port Hope STP in Port Hope. Maps of the Humber and Port Weller STP's are shown in the Materials and Methods section of the Phase I report.
Maps of the Ingersoll and Port Hope STP's are given in Appendix A.

a) Ingersoll sewage treatment plant

At the time of sampling, the Ingersoll STP was participating in a study being conducted on the effect of chlorine disinfection on bacteria. The first set of samples for virological analysis was collected in December, 1979, when chlorination of the final effluent was shut off. The second set was collected from this plant in January, 1980, when the effluent was being chlorinated.

Forty-litre grab samples were collected from the sewage outfall as well as from upstream and downstream stations in the receiving waters (see Appendix A). In addition, 48-hour gauze-pad samples were collected from both influent and effluent conduits of the treatment plant.

b) Port Hope sewage treatment plant

The effluent pipe from the Port Hope STP empties into Lake Ontario approximately 240 metres from shore at a depth of approximately 4 metres. Forty-litre grab samples were collected from the surface waters

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of Lake Ontario in the plume directly above the discharge from the effluent pipe, and from the chlorine contact chamber. Gauze-pads were immersed in the sewage effluent for 48 hours before being collected and processed.

c) Humber sewage treatment plant

The Humber STP was sampled on three occasions during April and May, 1980. The plant was not chlorinating the effluent on the first two sampling occasions, but effluent chlorination was being employed when the final set of samples was collected.

The effluent pipe discharges into Lake Ontario approximately 800 metres from shore at a depth of approximately 9 metres. Forty-litre grab samples were collected from the surface waters in the plume directly above the mouth of the effluent pipe, and from sampling sites approximately 400 metres east and 100 metres west of the discharge. In addition, gauze-pad samples were collected from the influent conduit and from the secondary settling tank prior to chlorination.

d) Port Weller sewage treatment plant

The Port Weller STP and its receiving waters were sampled on nine occasions during the summer of 1980.

The effluent from the STP empties into the Welland Canal, approximately two or three kilometres from the mouth of the canal. Grab samples were collected from the mixing zone (plume) at the end of the discharge pipe from the STP, from an upstream station approximately 200 metres from the discharge pipe, and from a sampling station approximately 2000 metres downstream from the outfall.

Gauze-pad samples were collected from the influent sewage conduit, and from the discharge from the chlorine contact chamber.

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RESULTS

A. CONCENTRATION AND RECOVERY OF VIRUSES FROM EXPERIMENTAL SAMPLES USING DUOFINE MEMBRANE FILTRATION

The capability of the filtration method to recover viruses from large volumes (40 L) of water was determined through experiments with type 1 (Sabin) poliovirus. The percentage of virus recovered from deionized water ranged from 14 to 141 percent (Table 1). In one experiment using lake water, 70 percent of the seeded virus was recovered. The results indicate that the filtration method employed was capable of recovering experimentally seeded type 1 poliovirus.

B. RECREATIONAL WATER AND SEDIMENTS

1. Beaches on the Great Lakes

A total of 20 water samples and 11 sediment samples was collected and analyzed for viruses, from selected bathing beaches on the Great Lakes (Table 2). No viruses were isolated from any of the samples.

2. River samples - Conservation area

The results of the virological analyses of surface water samples collected from Boyd and Clairville Conservation areas are summarized in Table 3.

A total of 7 samples was analyzed, and the presence of virus in three of these samples has been confirmed. Virus particles were isolated from the Boyd Conservation area on July 20, 1980, and from Clairville on July 20, 1980 and August 10, 1980.

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TABLE 1. Recovery of Poliovirus from 40 L Volumes of Water by Duofine Membrane Filtration

Sample	Experiment No.	Input Virus ($TCID_{50}$/ml)	Virus Recovered ($TCID_{50}$/ml)	% Virus Recovered
Deionized Water	1	1.26×10^4	1.78×10^4	141
	2	5.60×10^3	1.01×10^3	18
	3	3.20×10^2	1.70×10^2	53
	4	1.78×10^2	2.50×10^1	14
Lake Water	1	1.26×10^4	8.81×10^3	70

TABLE 2. Virological Analysis of Water and Sediment Samples from Bathing Beaches on the Great Lakes

	Site	Date Sampled	Results	
			40 L Water Samples	Sediment Sampled
1.	Jones Beach Lake Ontario	Oct. 10/79 Sept. 26/79	- N.D.[a]	- -
2.	Confederation Park Lake Ontario	Sept. 26/79	-[b]	-
3.	Hanlan's Point Beach Lake Ontario	Oct. 1/79	-	-
4.	Breakwater Beach Lake Ontario	Oct. 1/79	-	-
5.	Lakeside Park Lake Ontario	Oct. 10/79	-	-
6.	Wasaga Beach #2 Georgian Bay	Oct. 16/79 July 13/80 Aug. 4/80	- - -	- N.D. N.D.
7.	Wasaga Beach #5 Georgian Bay	Oct. 16/79	-	-
8.	Ipperwash Beach Lake Huron	Oct. 21/79	-	-
9.	Grand Bend Beach Lake Huron	Oct. 21/79 July 13/80 July 20/80	- - -	- N.D. N.D.
10.	Iroquois Beach Prov. Park Lake Erie	Oct. 21/79	-	-
11.	Municipal Beach Lake Ontario	July 27/80 Aug. 4/80 Aug. 10/80 Aug. 27/80 Sept. 9/80 Sept. 25/80	- - - - - -	N.D. N.D. N.D. N.D. N.D. N.D.

a) N.D. = Not Done
b) - = Negative (no virus detected).

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TABLE 3. Virological Analysis of Surface Waters From Conservation Areas

Site	Date Sampled	Presence of CPE in Cell Culture	Confirmation of Virus by Electron Microscopy
Boyd Conservation Area	6/7/80	-[b]	N.D.[a]
	13/7/80	-	N.D.
	20/7/80	+[c]	+
Clairville Conservation Area	29/6/80	-	N.D.
	6/7/80	-	N.D.
	20/7/80	+	+
	10/8/80	+	+

[a] N.D. = Not Done

[b] - = Negative

[c] + = Positive

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C. SEWAGE TREATMENT PLANTS (STP)

1. Ingersoll STP

The results of the virological analyses of samples collected from Ingersoll STP and its receiving waters are presented in Table 4.

Viruses were isolated from all five samples collected while the STP was not chlorinating the effluent, and also from all five samples collected after the plant had resumed chlorination.

2. Port Hope STP

The results of the analyses of samples collected from the Port Hope STP are presented in Table 5.

Viruses were isolated from grab samples of the unchlorinated effluent and from the plume in the surface waters of Lake Ontario above the discharge pipe. The one sample collected from the chlorinated effluent did not reveal the presence of virus.

3. Humber STP

Results of the analyses of samples collected from the Humber STP and receiving waters are presented in Table 6.

Virus particles were recovered from the surface waters of Lake Ontario in the plume immediately above the discharge from the effluent pipe. The plant was not chlorinating the effluent at the time these samples were taken. No viruses were recovered from these waters in the sample taken while chlorination was being practised.

No viruses were recovered from samples taken from surface waters outside the effluent plume. This may be attributable to the dilution factor presented by the lake and to previously discussed limitations of the methodology.

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TABLE 4. Virological Analysis of Sewage and Receiving River Water Samples from Ingersoll Sewage Treatment Plant

	Effluent not Chlorinated	Effluent Chlorinated
Influent Sewage (gauze pad)	Enterovirus	+[b]
Effluent Sewage		
1. (gauze pad)	Reovirus	+
2. (40 L grab)	Enterovirus (Polio 2)[a]	+
Receiving River Water		
1. Upstream (40 L grab)	Reovirus	+
2. Downstream (40 L grab)	Reovirus	+

[a] Confirmed by neutralization test with specific antiserum

[b] Virus group unidentified - Virus presence confirmed by electron microscopy.

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TABLE 5. Virological Analysis of Samples from the Port Hope Sewage Treatment Plant

Date	Chlorination	Sample Type	Location	Results
16/7/80	off	1. Gauze-Pad	Effluent Pipe	+[a]
		2. 40 L Grab	Effluent Pipe	+
4/9/80	on	1. 40 L Grab	Effluent Pipe	-
17/9/80	off	1. Gauze-Pad	Effluent Pipe	+
		2. Gauze-Pad	Effluent Pipe	+
		3. 40 L Grab	Effluent Pipe	+
		4. 40 L Grab	Outfall (Plume)	+

[a] + = virus presence confirmed by electron microscopy.

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TABLE 6. Virological Analysis of samples from the Humber Sewage Treatment Plant

Date	Chlorination	Sample Positive for Virus [a]				
		Outfall Plume (40 L)	East of Outfall (40 L)	West of Outfall (40 L)	Influent Sewage (Pad)	Effluent Sewage (Pad) (In 2° Settling Tank)
23/4/80	off	+	-	-	+	-
7/5/80	off	+	-	-	+	-
21/5/80	on	-	-	-	+	+ (not chlorinated)

[a] Confirmed by electron microscopy.

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4. Port Weller STP

The results of the virological analyses of samples collected from the Port Weller STP and its receiving waters are presented in Table 7.

Viruses were recovered from all eight gauze-pad samples taken from the influent sewage. Three of six grab samples taken from the outfall plume of the chlorinated effluent were positive for viruses. No viruses were recovered from any of the downstream samples collected 2000 metres below the outfall. Virus was recovered from one sample collected upstream from the outfall pipe.

D. SUMMARY

1. Virological analysis of surface waters

A total of 66 surface water samples was collected and analyzed. The results of these analyses are presented in Table 8.

Viruses were recovered from three of the thirty lake water samples analyzed. All three positive samples were collected from sewage outfall plumes.

Viruses were recovered from 33% (12 of 36) of the river samples analyzed. All nine samples collected downstream from the Port Weller STP were negative.

Eleven samples were analyzed from the Humber and Thames rivers and viruses were recovered from seven (64%) of these.

2. Virological analysis of samples collected from sewage treatment plants

A total of 42 samples collected from STP influents and effluents was analyzed in this study. The results are presented in Table 9.

Each of the 13 gauze-pad samples taken from influent sewage revealed the presence of viruses. Viruses were recovered from 67% (8 of 12) of the effluent pad samples.

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TABLE 7. Virological Analysis of Samples from Port Weller Sewage Treatment Plant

Date	Chlorination	Samples Positive for Virus [a]				
		Influent Sewage (Pad)	Effluent Sewage (Pad)	Outfall (40 L)	Upstream (200 m) (40 L)	Downstream (2000 m) (40 L)
30/4/80	off	+	+	+	-	-
14/5/80	off	+	N.D.[b]	-	-	-
29/5/80	on	+	+	+	-	-
27/7/80	on	+	N.D.	-	+	-
4/8/80	on	+	N.D.	N.D.	-	-
10/8/80	on	N.D.	N.D	+	-	-
27/8/80	on	+	N.D.	-	-	-
9/9/80	on	+	-	-	N.D.	-
25/9/80	on	+	-	+	-	-

a Confirmed by electron microscopy

b N.D. = Not done.

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TABLE 8. Virological Analysis of Surface Waters

Surface Water Type		Number of Samples Tested	Number of Samples Positive	Percent Positive
A. Lake				
1. Bathing Beach Site		20	0	
2. Near Sewage Outfall				
a) In Plume		4	3	
b) Outside Plume		6	0	
	Total	30	3	10
B. River				
1. Conservation Area Site		7	3	
2. Upstream of STP		10	3	
3. Downstream of STP				
a) In Plume		8	4	
b) Outside Plume		11	2	
	Total	36	12	33

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TABLE 9. Virological Analysis of Sewage Treatment Plant Samples

Sample Type	Chlorination On			Chlorination Off		
	No. of Samples	No. Positive for Virus	% Positive	No. of Samples	No. Positive For Virus	% Positive
Influent Sewage (Pad)	8	8	100	5	5	100
Effluent Sewage (Pad)	4	2	50	8	6	75
Effluent Sewage or Plume (40 L)	9	4	44	8	7	88
Receiving Water						
A. Upstream	8	2	25	5	1	20
B. Downstream	9	1	11	5	1	20

Two of four pad samples collected from chlorinated effluents were positive for viruses, while four of nine (44%) grab samples of chlorinated effluent were positive. A sample collected from the secondary settling tank of the Humber STP was positive for virus. The STP was chlorinating the effluent but the sample was collected before the chlorine contact pipe.

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DISCUSSION

Viruses in water act as colloidal hydrophilic particles that can interact with other charged particles and solid surfaces. Depending upon the pH of the water, they are either positively or negatively charged. At pH values normally found in natural waters, viruses behave as negatively charged particles with the ionogenic groups present on the virus governing the adsorptive properties of the virus. Since electrostatic attraction plays a major role in the adsorption of viruses to filters, samples are "conditioned" in order to enhance virus adsorption. Cations, in the form of EBSS, are added to the water to decrease the negative charge on membrane filters thereby enhancing virus adsorption to the surface of the membrane. The cations may also form a salt bridge to help bind the virus. Lowering the pH of the sample to 5.5 decreases the negative charge of the virus (the virus is positively charged at pH values below its isoelectric point), and enhances the electrostatic attraction between virus and filter. At lower pH's (3.0 - 3.5), virus adsorption to filters may be further enhanced, but certain viruses may be inactivated. The same factors govern the elution of the viruses from filters. At high pH's the virus bears a stronger negative charge and has little affinity for the negatively charged filter. Fetal calf serum (FCS) is added to the eluting fluid to aid in the desorption process. The proteins in the FCS compete with the viruses for binding sites on the filter and dislodge the virus particles. Depending on the type of eluting fluid, pH's of 9 to 11.5 are commonly used. Since certain viruses may be rapidly inactivated at pH 11.5, the time of exposure to this pH is kept to a minimum.

As shown in Table 1, the filtration method employed in this study was capable of recovering experimentally seeded type 1 poliovirus. Since this virus has adsorptive properties similar to those of some other enteroviruses, the method was deemed suitable for the qualitative examination of surface waters. However, the method still has numerous limitations. Over 100 types of enteric viruses are known

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to be excreted in human feces. Table 10 lists the major groups of enteric viruses which have been isolated from raw sewage or from fecal material from infected persons. Approximately two-thirds of these viruses cannot be recovered in vitro using currently available methods. This severely limits the recovery spectrum of any method employed. Estimates of the actual efficiency of virus concentration methods for recovering all enteric viruses range from ten to twenty percent. Therefore, the absence of virus from a field sample does not necessarily indicate that the sample contained no virus, but only that no virus could be detected. The isolation of any enteric virus from an environmental sample indicates the presence of fecal contamination and a potential health hazard, as undetectable pathogenic viruses (e.g. hepatitis A, rotavirus) may be present.

A. RECREATIONAL WATER AND SEDIMENTS

1. Beaches on the Great Lakes

No viruses were isolated from any of the twenty water samples or the eleven sediment samples analyzed from selected bathing sites on the Great Lakes. Viral pollution of surface waters may be considered a fairly transient phenomenon dependent upon input concentration, dilution factors, flow characteristics of the water course, and the survival rate of the viruses. Figure 1 illustrates possible modes of enteric virus transmission through the water route. The dilution factor presented by the Great Lakes greatly reduces the concentration of input virus and since there is relatively little water turbulence in the lakes, virus particles would probably sediment out fairly rapidly.

2. River samples - Conservation areas

The isolation of virus from three of seven (43%) Humber River samples is indicative of the increased susceptibility of bathing areas on rivers, as opposed to beaches on lakes, to viral contamination. The dilution factor presented by a river is usually much less than that of a lake, and the flow of a river aids in keeping

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TABLE 10.[a] Human Enteric Viruses that may be Present in Water[b]

Virus group	Number of types	Disease caused
Enteroviruses		
Poliovirus	3	Paralysis, meningitis, fever
Echovirus	34	Meningitis, respiratory disease, rash, diarrhea, fever
Coxsackievirus A	24	Herpangina, respiratory disease, meningitis, fever
Coxsackievirus B	6	Myocarditis, congenital heart anomalies, rash, fever, meningitis, respiratory disease, pleurodynia
New enterovirus type 68-71	4	Meningitis, encephalitis, respiratory disease, acute hemorrhagic conjunctivitis, fever
Hepatitis type A (probably an enterovirus)	1	Infectious hepatitis
Gastroenteritis type A (probably an enterovirus)	2	Epidemic vomiting and diarrhea, fever
Rotavirus (Reovirus family)	- 1	Epidemic vomiting and diarrhea, chiefly of children
Reovirus	3	Not clearly established
Adenovirus	30	Respiratory disease, eye infections
Norwalk agent	1	Gastroenteritis

a) Taken from Melnick (2).

b) Other stable viruses that might contaminate water are -
SV40-like papovaviruses, which appear in the urine. The JC subtype is associated with progressive multifocal leukoencephalopathy.
Creutzfeld-Jacob disease virus. Like scrapie virus, the C-J virus resists heat and formaldehyde. The virus causes a spongiform encephalopathy, characterized by severe progessive dementia and ataxia.
Parvoviruses. The adeno-associated satellite viruses that are excreted in stools of children survive heating for one hour at $60^{o}C$.

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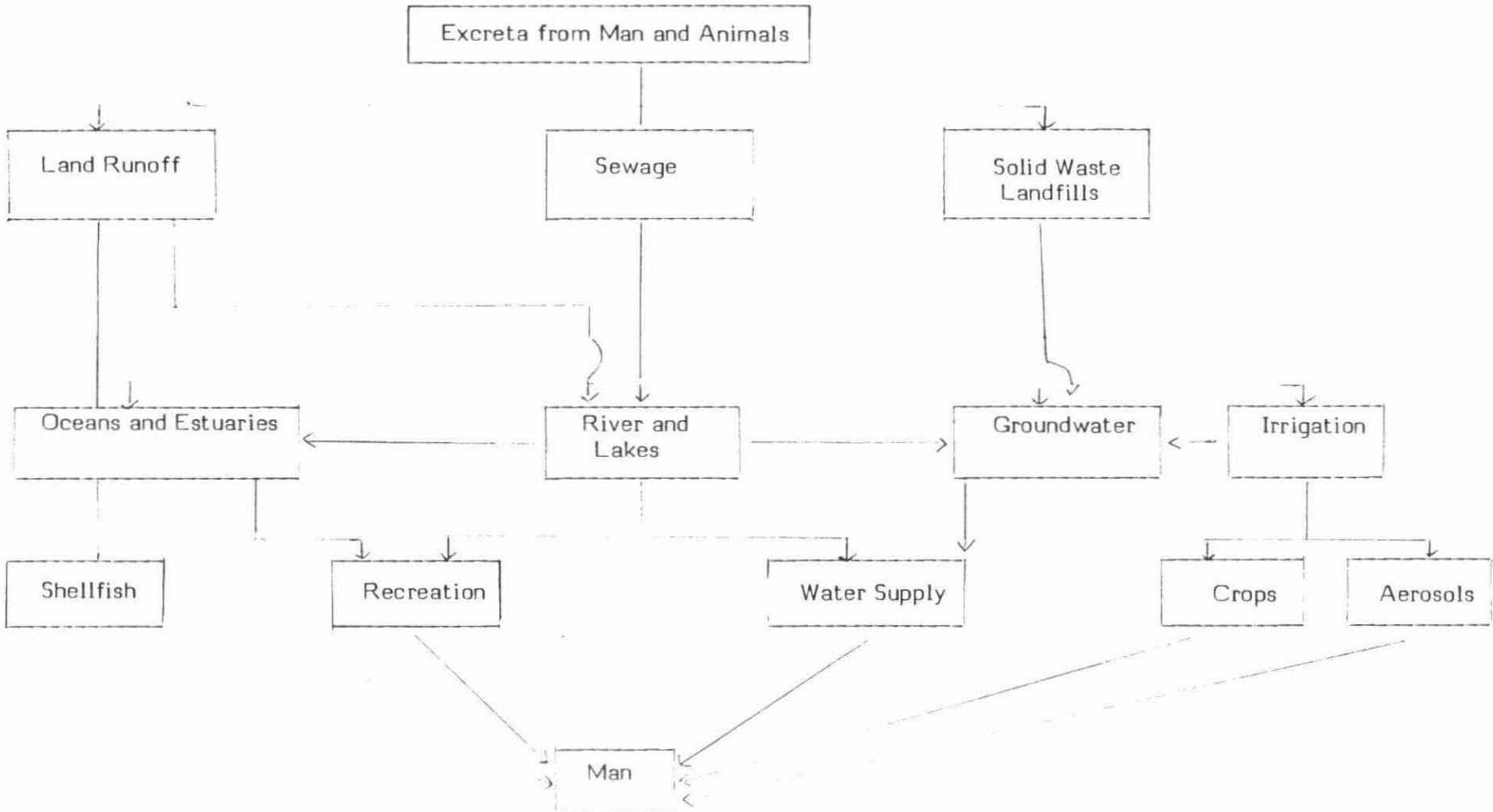

Fig. 1. Possible modes of enteric virus transmission by water (9).

viruses suspended for longer periods of time. Consequently, the source of the virus could be many miles upstream. Although it is possible that swimmers themselves may provide a source of inoculum of virus, this is not expected to be a major contributing factor.

B. SEWAGE TREATMENT PLANTS

As shown in Table 9, viruses were recovered from all types of STP samples. All of thirteen raw sewage pad samples were virus positive, as were eight of twelve effluent sewage pad samples. In addition, eleven of seventeen effluent sewage or sewage plume grab samples were positive for virus. These results confirm that STP effluents are a major source of enteric viruses in the environment.

The results also indicate that viruses could readily be recovered from STP effluents even when chlorination was being practised. This is not surprising, since many authors have reported that enteric viruses may easily survive secondary sewage treatment and chlorination (1, 2, 3, 4, 10, 11). The fate of viruses during sewage treatment is currently the subject of numerous studies. Since viruses in solution behave as charged particles, many viruses are removed in the sedimentation processes. Viruses are thereby concentrated in the sludge where they may continue to pose a health hazard. Viral particles that are not sedimented out are discharged in the effluent. Chlorination of the effluent may significantly reduce the number of infectious viral particles in the effluent, but the potential effectiveness of chlorine disinfection is not normally realized. This is due to a number of factors:

1) The most effective chlorine species for the inactivation of viruses is hypochlorous acid (HOCl). Hypochlorite ion (OCl^-) and chloramines are not as effective against viruses. Chlorine present in water as HOCl or OCl^- is defined as free available chlorine, with the predominating chlorine species primarily dependent upon the pH of the water. At pH < 6, hypochlorous acid

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predominates while at pH > 9.5, hypochlorite is the predominant form. In most sewage effluents, chlorine species combine with ammonia and organics to form chloramines which are not effective virucidal agents. Therefore, unless the sewage effluent is essentially free of ammonia and organics, most of the chlorine will not be in a highly virucidal form.

2) The rate of inactivation of different viruses by chlorine is highly variable. Under ideal conditions (i.e. "clean" water and chlorine in HOCl form), 99.99% viral inactivation may be obtained in 30 minutes with 0.5 ppm hypochlorous acid. In practice, the exposure time to chlorination is much less, often being less than ten minutes.

3) Aggregated viral particles, and viral particles adsorbed to particulate matter, have been found to be much more resistant to chlorine disinfection than single virus particles. Most ($\leq$ 90%) viruses in sewage are particle-associated. This further minimizes the effectiveness of chlorine disinfection.

The findings of this study provide further confirmation that viruses are released into the environment through sewage effluents. Viruses were recovered from the chlorinated effluent of two of four sewage treatment plants studied. This is of particular concern when the receiving water of an effluent is an inland river, since the viruses may be kept in suspension due to water movement, and the dilution factor is usually much less than that of large lakes. The viruses may survive for months and could pose a risk to all users of the water downstream.

Viruses in water pose a hazard to bathers and to consumers of the water. A recent epidemiological study by Cabelli found a significant incidence of swimming-associated illness in bathers when the bathing surface waters were well within current microbiological standards (17). The etiological agent was not identified, but from the evidence gathered, Cabelli postulated that the agent was a highly infectious virus, quite possibly rotavirus. It is known that rotaviruses may be present in very high concentrations (> 10^9 particles/g) in the feces of infected

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individuals, but since methods have not been developed to recover rotaviruses from surface waters, the extent of surface water contamination with this virus is not known.

Rotavirus appears to be one of the leading causes of infantile acute gastroenteritis in the world. Although gastroenteritis is a more serious problem in undeveloped countries (approximately 5 to 18 million deaths/yr. in Africa, Asia, and Latin America), it is still of major concern in developed countries (11). Between May 1971 and March 1977, there were 21 fatal cases of infantile acute gastroenteritis in Toronto, Canada. Rotavirus particles were isolated from all of these cases (11).

It is evident that viruses can survive sewage treatment. The methods used in this study were qualititative, capable of the presence-absence detection of certain viruses. In order to understand the fate of viruses in the environment, quantitative methods should be developed and employed for studying the virucidal effectiveness of sewage treatment plant processes and for the analysis of larger volumes of surface water. Since viruses are released into the waterway, they may be present in raw water drawn for processing into drinking water. Chlorine-resistant strains of enteroviruses have recently been recovered from chlorinated drinking water (18). Virological techniques for analyzing large volumes of water should be applied to the analysis of drinking water to ensure that current procedures for processing water are adequate for the removal of viruses.

No attempt has been made to link the virological data to the epidemiological data presented in the other part of this report. The amount of virological data is too small for interpolation into epidemiological correlations. In addition, such analyses are not possible without extensive serological typing of the isolated viruses, which was beyond the scope of this study.

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RECOMMENDATIONS

1. All virus isolates should be identified and serotyped where possible. This work is currently underway and the results will be summarized in the Phase III - Final Report.
2. The isolation of viruses from both conservation areas examined indicates the need for more extensive studies to assess the risk posed by viruses in such areas in Ontario.
3. Quantitative methods need to be developed for the recovery and isolation of a wider range of enteric viruses, with the development of rotavirus and Norwalk agent recovery methodologies given high priority. The development of automated sampling techniques for analyzing large volumes of surface or drinking water should also be pursued.
4. Quantitative methods for the recovery of enteric viruses should be employed to study the efficiency of sewage treatment plant practices for removing or inactivating virus.
5. Many of the receiving waters for sewage effluents in Ontario are subsequently used as sources of raw water for potable water processing. The extent of viral contamination of raw water at treatment plant intakes needs to be assessed to ascertain what risk may be posed by ineffective treatment of this water.
6. Raw drinking water supplies are normally treated before consumption. These treatment procedures should be examined in order to ascertain their virus removal and inactivation efficiency.

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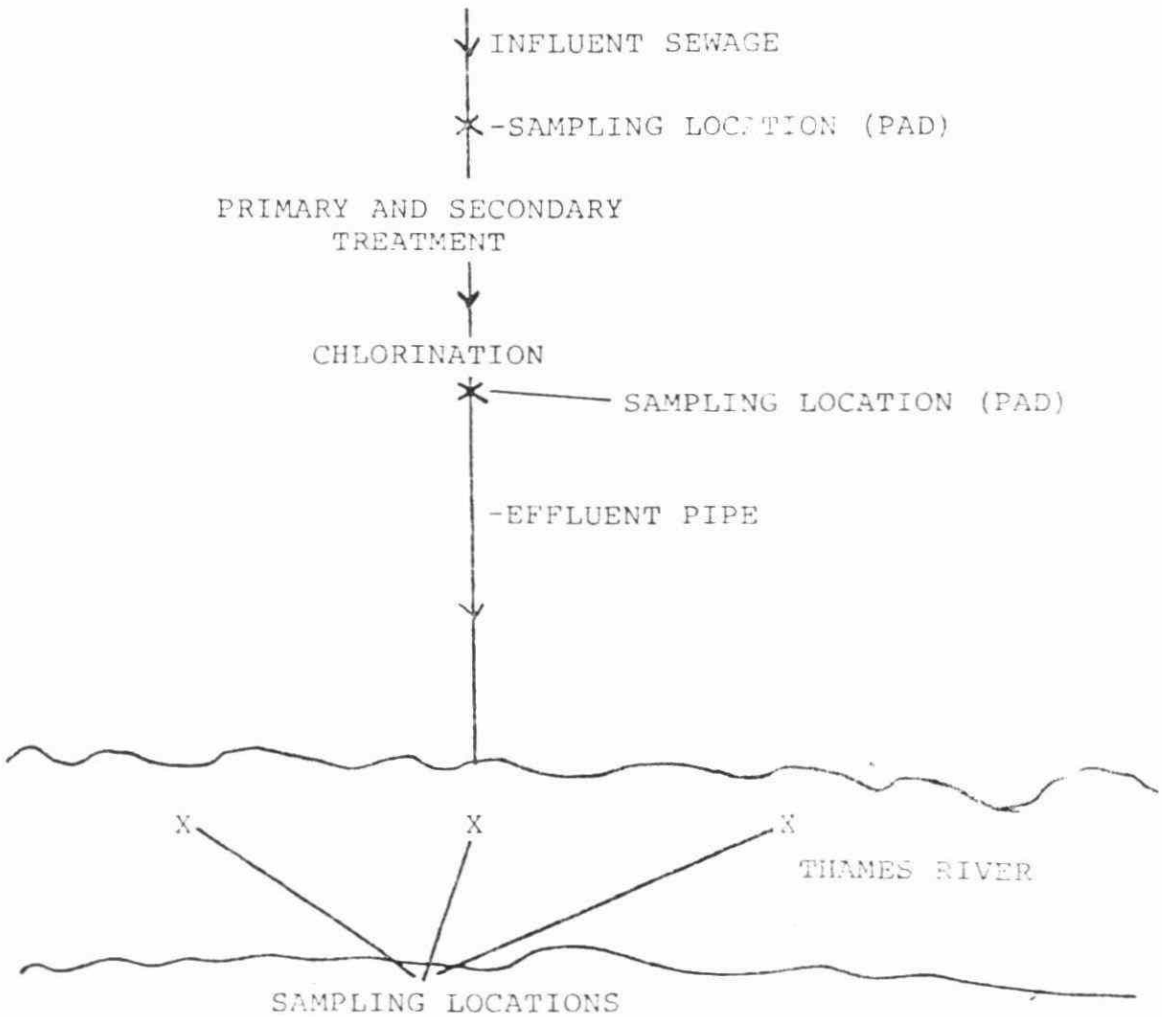
APPENDIX A

SAMPLING LOCATIONS - INGERSOLL SEWAGE TREATMENT PLANT



SAMPLING LOCATIONS - PORT HOPE SEWAGE TREATMENT PLANT